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In vivo microdialysis and electroencephalographic activity in freely moving guinea pigs exposed to organophosphorus nerve agents sarin and VX: analysis of acetylcholine and glutamate

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Abstract Organophosphorus nerve agents such as sarin (GB) and VX irreversibly inhibit acetylcholinesterase, causing a buildup of acetylcholine (ACh) in synapses and neuromuscular junctions, which leads to excess bronchial secretions, convulsions, seizures, coma, and death. Understanding the unique toxic characteristics of different nerve agents is vital in the effort to develop broad spectrum medical countermeasures. To this end, we employed a repeated measure multivariate design with striatal microdialysis collection and high-performance liquid chromatography analysis to measure changes in concentrations of several neurotransmitters (ACh, glutamate, aspartate, GABA) in the same samples during acute exposure to GB or VX in freely moving guinea pigs. Concurrent with microdialysis collection, we used cortical electrodes to monitor brain seizure activity. This robust double multivariate design provides greater fidelity when comparing data while also reducing the required number of subjects. No correlation between nerve agents' propensity for causing seizure and seizure-related lethality was observed. The GB seizure group experienced more rapid and severe cholinergic toxicity and lethality than that of the VX seizure group. Seizures generated from GB and VX exposure resulted in further elevation of ACh level and then a gradual return to baseline. Glutamate levels increased in the GB, but not in the VX, seizure group. There were no consistent changes in either aspartate or GABA as a result of either nerve agent. These observations reinforce findings with other nerve agents that seizure activity per se contributes to the elevated levels of brain ACh observed after nerve agent exposure.

Keywords Acetylcholine · Acetylcholinesterase · Choline · Electroencephalogram · γ-Aminobutylic acid (GABA) · Glutamate · Guinea pig · In vivo microdialysis · Nerve agents · Organophosphorus compounds · Sarin · Seizure activity · VX

Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
AMN	Atropine methyl nitrate
Asp	Aspartic acid; aspartate
ChE	Cholinesterase
CNS	Central nervous system
EAA	Excitatory amino acid
EC	Electrochemical
ED_{50}	Median effective dose
EEG	Electroencephalographic
Glu	Glutamic acid; glutamate
GABA	γ-aminobutyric acid
GB	Sarin; isopropyl methylphosphonofluoridate
GD	Soman; pinacolyl methylphosphonofluoridate
HPLC	High-pressure liquid chromatography
im	Intramuscular
LD_{50}	Median lethal dose
NS	No seizure
NT	Neurotransmitter
OP	Organophosphorus compound
OPA	o-phthalaldehyde
PNS	Peripheral nervous system
S	Seizure
SAL	Saline

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sc	Subcutaneous
VX	o-ethyl S-(2-(diisopropylamino)ethyl) methylphosphonothioate

Introduction

Chemical warfare nerve agents such as sarin (GB) and VX are organophosphorus (OP) cholinesterase (ChE) inhibitors. Their major cause of toxicity is the irreversible inhibition of ChE, in particular acetylcholinesterase (AChE), the enzyme responsible for breaking down the neurotransmitter (NT) acetylcholine (ACh), which leads to an accumulation of extracellular ACh within both the central (CNS) and peripheral nervous systems (PNS) and at neuromuscular junctions. The subsequent hyperactivities of the cholinergic system can produce a sequence of toxic signs such as tremors, convulsions/seizures, bronchospasm, excess bronchial secretions, respiratory distress, coma, and death (Taylor 2001).

During the past two decades, a series of pharmacological mechanistic studies (Shih et al. 1991, 2003; McDonough and Shih 1993, 1997; Shih and McDonough 1997, 2000) has established a model for the progression of events following nerve agent-induced seizures. The progression is divided into three phases: an early cholinergic phase, lasting from the time of exposure to a short time after seizure onset; a transitional phase of progressively mixed cholinergic/non-cholinergic modulation, which begins shortly after seizure onset and lasts until about 40 min after seizure onset; and finally, a predominantly non-cholinergic phase starting at about 40 min after seizure onset in which excitatory amino acids (EAA), in particular glutamate (Glu) and aspartate (Asp), maintain seizure activity. Excitotoxicity due to extended periods of elevated extracellular Glu causes cell death, and brain damage will result from prolonged seizure activity following nerve agent exposure.

It has been speculated that excess ACh in the synapse binding presynaptic autoreceptors and downregulating further ACh release may contribute to physiological inhibition of seizure activity. However, it is likely that additional neurotransmitters also contribute to blocking seizure initiation as part of the neurochemical stress response. Tonduli et al. (1999) administered a seizurogenic dose of the OP nerve agent soman (GD) to rats while recording electroencephalogram (EEG) for power spectrum analysis and found that only rats that lacked an increase in the gamma band experienced seizure. The gamma band is associated with stress, attentional focus, and noradrenergic pathways, which led Tonduli and colleagues to conclude that the observed increase in the gamma band represented an adaptation response to the increased excitation from nerve agent exposure. The neurochemical perturbations and peripheral toxic effects resulting from nerve agent exposure preceding and following seizure initiation are a source of stress that the animal has no means of directly attenuating. Uncontrollable stress has been shown to result in decreased chloride uptake by γ-aminobutyric acid A ($GABA_A$) receptors in frontal cortex and amygdala, and chewing (an anxiety-induced active coping behavior in rodents) has been shown to restore chloride uptake (Martijena et al. 2002). Excessive chewing is commonly observed in rodents exposed to nerve agent, but it has not been investigated in the context of seizure occurrence. However, increasing the affinity of $GABA_A$ receptors for GABA is the main pathway of benzodiazepines, which have demonstrated rapid termination of nerve agent-induced seizures (Shih et al. 2003). Here, we investigate the factors contributing to seizure initiation by examining neurochemical and EEG changes at nerve agent doses known to elicit seizure and non-seizure responses.

Changes in both extracellular NT levels measured by in vivo microdialysis techniques and total NT levels measured in brain homogenates following exposure to seizure-inducing doses of a nerve agent have been documented in animal studies. Upon exposure to GD, total brain ACh levels increase immediately (Shih 1982; Fosbraey et al. 1990; Shih and McDonough 1997), but return to extracellular baseline levels within 90 min of seizure onset (Lallement et al. 1992). Late increases in extracellular Glu have also been reported in numerous brain regions following GD-induced seizure onset (Lallement et al. 1991a, b, 1992b; Wade et al. 1987; Shih and McDonough 1997). These data, taken together, support the idea that there is a triphasic NT model for onset and progression of seizures and subsequent brain damage upon acute exposure to nerve agent (McDonough and Shih 1997). However, a systematic study of the time-course changes in critical NT levels in the brains of animals exposed to nerve agents other than GD has not yet been reported. Such studies may lead to identification of intrinsic factors differentiating the toxic consequences of these chemical threat agents. For these reasons, this experiment assessed the time-dependent changes in extracellular concentrations of ACh, Glu, Asp, and GABA in the striatum of guinea pigs challenged with the nerve agents GB and VX.

GB and VX were chosen for this study because of their key structural differences (Fig. 1) and because they have reportedly been employed during warfare and terrorist attacks (Malloy 2000; Szinicz 2005; Smart et al. 2008). Compared to GB, the chemical structure of VX is larger and carries a charge (circulates in vivo as a protonated amine), which results in delayed passage across the

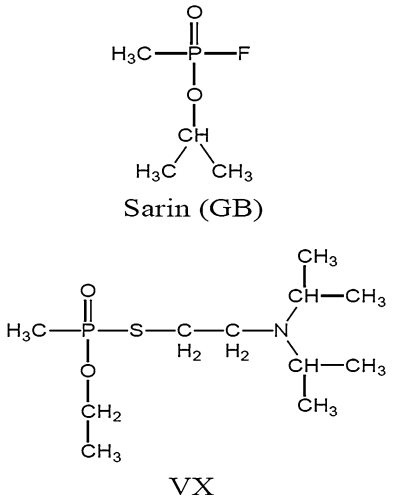

Fig. 1 Chemical structures of the nerve agents sarin and VX

blood–brain barrier as demonstrated by time-course AChE inhibition data from within the brain compared with inhibition profiles from peripheral tissues (Shih et al. 2005) and the delayed signs of intoxication (e.g., onset of EEG seizure activity) when compared with GB and other nerve agents (Shih et al. 2003, 2007). In addition, administration of 1.0 × LD_{50} nerve agent will not always result in motor convulsions and brain seizures (Shih 1982; Lallement et al. 1992a; Tonduli et al. 1999). We have taken advantage of this fact to produce animals that received similar challenge doses of nerve agent, but only a subset of which go on to develop sustained seizures, in order to evaluate the differential contribution of the seizure activity to any observed changes in NT levels or lethality.

Materials and methods

Animal welfare

The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council 1996) and the Animal Welfare Act of 1966 (P.L. 89-544), as amended. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Subjects

Male Hartley guinea pigs weighing 250–350 g were purchased from Charles River Labs (Kingston, NY). They were individually housed in polycarbonate cages in temperature (21 ± 2°C) and humidity (50 ± 10%) controlled quarters that were maintained on a 12-h light–dark schedule (with lights on at 0600 h). Laboratory chow and filtered tap water were freely available whenever the animals were in their home cages. Animals were allowed to acclimate for 1 week prior to experimentation.

Although rat and mouse models have been utilized for nerve agent studies, the guinea pig is considered to be a more suitable animal model (Inns and Leadbeater 1983). Unlike rats and mice, guinea pigs do not possess high levels of carboxylesterase, which binds nerve agents such as GB or GD and reduces the amount of these agents available to inhibit AChE (Maxwell et al. 1988). In addition, guinea pigs are more similar to non-human primates in their response to pyridostigmine bromide pretreatment for protection against nerve agents (Maxwell et al. 1987, 1988). Guinea pigs have been used previously to study protection against acute toxicity, prophylactic and therapeutic treatment of seizures, and pathology following nerve agent exposure (Shih et al. 1996, 2003, 2007, 2009; McDonough and Shih 1997; Shih and McDonough 1999; Atchison et al. 2004).

Materials

Neostigmine bromide, acetylcholine chloride, choline chloride, L-glutamic acid, L-aspartic acid, GABA, glucose, sodium chloride, potassium chloride, magnesium chloride, calcium chloride, 1-octanesulfonic acid sodium salt, atropine methyl nitrate (AMN), monobasic sodium phosphate, dibasic sodium phosphate, methanol (HPLC grade), acetonitrile (HPLC grade), 2-mercaptoethanol, and pentobarbital sodium were purchased from Sigma–Aldrich (St. Louis, MO). O-phthalaldehyde (OPA) and OPA diluent were purchased from Pickering Laboratories (Mountainview, CA). Buprenorphine HCl was purchased from Reckitt Benckiser Pharmaceuticals Inc. (Richmond, VA). Phosphoric acid (HPLC Grade) was purchased from Fischer Scientific (Fair Lawn, NJ). Sarin (GB; isopropyl methylphosphonofluoridate) and VX (o-ethyl S-(2-(diisopropylamino)ethyl) methylphosphonothioate) were obtained from the U. S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). Nerve agents and AMN were diluted in sterile saline prior to injection at a concentration to allow for an injection volume of 0.5 ml/kg. Nerve agents were administered subcutaneously (sc), and AMN was given through intramuscular (im) injection.

Surgery

Guinea pigs were anesthetized with isoflurane and surgically implanted with cortical screw electrodes and

microdialysis guide cannulae using standard aseptic surgical techniques (Shih and McDonough 2000). Anesthetized guinea pigs were placed in a Kopf stereotaxic frame, and stainless steel cortical EEG screws were placed approximately 3.0 mm lateral from midline and equidistant between bregma and lambda skull coordinates. The screws were attached to a miniature connector via stainless steel wires. Guide cannulae (15 mm in length; part no. MD2251; Bioanalytical Systems, W. Lafayette, IN) were targeted stereotaxically at the caudate nucleus (+11.4 mm anterior, +3.6 mm lateral, −4.6 mm ventral to the skull surface) based on the atlas of Luparello (1967) using a device defined zero coordinate (Kopf electrode angle calibrator [model 935]). Screws, wires, connector, and guide cannulae were then anchored to the skulls with dental acrylic. Guinea pigs received 0.07 ml of 0.15 mg/ml buprenorphine HCl (sc) prior to and following surgery for pain control. Subjects were allowed to recover for 6–10 days before the day of the experiment.

The striatum was chosen as the region for in vivo microdialysis collection for its position along the seizurogenic pathways and its relatively larger nuclei; the latter would reduce probe placement errors. The striatum accounts for most of the basal ganglia, which has been implicated in OP-induced seizure potentiation. It is innervated by glutamatergic excitatory afferent neurons from the neocortex and substantia nigra, which have been respectively identified as a seizurogenic area and an area vital for seizure propagation during nerve agent poisoning (Glenn et al. 1987), although the striatum itself has not been shown to be vital for seizure initiation or maintenance. The striatum is a relatively large structure (easily targeted stereotaxically) with high extracellular ACh concentration that could serve as an indicator of global cholinergic events. The high concentration of ACh in the striatum is due to its being almost entirely made up of cholinergic interneurons. This high level of ACh makes monitoring neuroactive levels of the amino acids of interest particularly challenging, since they may not be present in sufficient amounts under normal physiological conditions. Furthermore, Glu transporters on the microglia encapsulating the synapse are extremely efficient (Del Arco et al. 2003). Because microdialysis samples are collected from the extracellular space outside of the synapse, elevated levels of excitatory amino acids in the striatum may only be detected under conditions of extremely robust signaling from afferent regions. Data were interpreted with these characteristics of the striatum in mind.

Experimental procedure

On the day of the experiment, each animal was placed in an individual collection chamber (23 cm deep × 31 cm wide × 45 cm high). A brain microdialysis probe (0.34 mm OD, BAS BR-2 [part no. MD-2200]) was inserted into the guide cannula, and an EEG commutator was plugged into the miniature connector. The probe was perfused at a constant rate of 2.0 μl/min with an artificial cerebrospinal fluid (ACSF) containing 140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1.2 mM Na_2HPO_4, 0.27 mM NaH_2PO_4, 74 mM glucose, and 2 μM neostigmine Br (pH 7.4), and a 2.5-h normalization period was applied. After the normalization period, EEG recording was initiated along with the continuous collection of dialysate samples at 15-min intervals, resulting in a total volume of 30 μl in each vial. Basal NT levels were collected for 60 min prior to giving AMN (1.0 mg/kg, im). AMN is a muscarinic receptor antagonist that does not cross the blood–brain barrier. It provides prophylactic protection from peripheral cholinergic toxic effects during the experiment without interfering with pharmacological events within the brain (Chambers and Chambers 1989; Shih 1991; Maalouf et al. 1998). Thirty minutes after AMN, the subjects were injected (sc) with sterile saline or 1.0 × LD_{50} of GB (42 μg/kg) or VX (8 μg/kg). These LD_{50} doses of GB and VX are based on historical determinations of these values at our Institute in this species by this route of administration. The experiment was continued for 255 min (17 samples) after nerve agent exposure. After collection, the dialysate samples were frozen and kept at −20°C until analysis for NTs. At approximately 24 h after exposure, survivors were deeply anesthetized with pentobarbital sodium (75–100 mg/kg, ip) and perfused with saline followed by 10% neutral buffered formalin for verification of probe location. Results from subjects with inaccurate probe placement were not included.

Electroencephalogram

Seizure occurrence was recorded via cortical EEG. EEG recordings and dialysate sampling were started simultaneously. EEG signals were captured using QND software (version 3.1, Neurodata Inc, Pasadena, CA) on a Macintosh personal computer. The analog signal was amplified with a NE-4 amplifier (Neurodata) before being processed on the Neurodata digital signal processor board. Onset of seizure activity was characterized by continuous spiking activity accompanied by an increase in the power spectrum greater than two times the baseline sustained for a period of not less than 10 s (Shih and McDonough 2000).

ACh and choline (Ch) analysis

Prior to ACh and choline analysis, 10 μl of the sample was dispensed into a separate vial for amino acid analysis. ACh and Ch levels within dialysate samples were quantified

using isocratic HPLC-EC. The pump (Model 582), detector (Coulachem III), analytical column (ESA ACH-250, 5 μm particle size, 250 × 3.2 mm; Part No. 70-4457), post-column reactor (ACH-SPR Part No. 70-0640), and analytical cell (Model 5040) were all obtained from ESA Biosciences, Inc. (Chelmsford, MA). Separation was achieved at room temperature. The mobile phase was composed of anhydrous dibasic sodium phosphate (100 mM), 1-octanesulfonic acid (2 mM), and Reagent MB (antimicrobial) from ESA Biosciences (50 μl/l) in deionized water (pH 8.0) and was delivered at a constant rate of 0.350 ml/min. The injection volume was 10 μl. To facilitate EC detection, a post-column reactor was utilized to convert ACh and Ch to hydrogen peroxide. The signal from the amperometric cell was delivered to a computer, and the digitized signal was analyzed with EZchrom elite software (Scientific Software, Pleasanton, CA). The system was calibrated using a set of standards ranging from 0.19 to 12.0 pmol of ACh and 0.31 to 20.0 pmol of Ch. We achieved good separation between ACh and Ch peaks, and the typical retention times were 5 min for Ch and 7.6 min for ACh. Ch is a naturally occurring ACh metabolite, but it is not active as a neurotransmitter. Ch concentrations were not evaluated for this study.

Amino acid analysis

Glu, Asp, and GABA levels within dialysate samples were quantified using isocratic HPLC-EC. The pump (Model 582), detector (Coulachem III), analytical column (ESA 90816, Shiseido capcell pak C18, type MG, 3-μm particle size, 75 × 3.0 mm), and analytical cell (Model 5011-A) were all obtained from ESA Biosciences, Inc (Chelmsford, MA). Separation was achieved at 35°C. The mobile phase was composed of anhydrous dibasic sodium phosphate (100 mM), 22% methanol, and 3.5% acetonitrile in deionized water (pH 6.75) and was delivered at a constant rate of 0.70 ml/min. A derivatization reaction was utilized to facilitate EC detection in which the autosampler would add 20 μl of OPA working reagent (2 ml OPA stock in 6 ml diluent) to 10 μl of sample, wait 2 min, and then inject the sample/OPA mixture. The injection volume was 17 μl. OPA stock contained 27 mg OPA in 9 ml diluent with 5 μl 2-mercaptoethanol. The signal from the coulometric cell was delivered to a computer, and the digitized signal was analyzed with EZchrom elite software (Scientific Software, Pleasanton, CA). The system was calibrated using a set of standards ranging from 1.56 to 100.0 pmol of Asp, 1.56 to 100.0 pmol of Glu, and 0.94 to 60.0 pmol of GABA. We achieved good separation, and the typical retention times were 2 min for Asp, 3 min for Glu, and 23.5 min for GABA.

Data analysis

Based on EEG analysis, animals were classified as showing sustained seizure (S; EEG activity that does not return to normal for the duration of the 255-min collection period after seizure onset), non-seizure (NS; no seizure onset), or unsustained seizure (after full seizure initiation EEG returns to normal during the collection period). Seizure and non-seizure classification were applied for NT analyses. The few animals that experienced unsustained seizure were excluded to avoid interference with seizure and non-seizure analysis. All tests were run using PASW Statistics 17 for Windows (SPSS Inc, Chicago, IL), and significance was set at $P < 0.05$.

Comparison of groups according to seizure occurrence and mortality within seizure groups was performed with a cross-tabulated chi-square test followed by pair-wise comparisons with Fisher's exact test. Comparison of seizure onset times was performed with a Student's T test.

The study design is a repeated measure with treatment (nerve agent seizure, nerve agent non-seizure, saline/saline, AMN/saline) as the independent variable and repeated measurements of variables from a single subject at regular intervals of time (15 min) as dependent variables. Multiple within subject factors (NTs) make up the dependent variables. In effect, there are several within subject factors (NTs) for each of the repeated measure dependent variables (time points), which is referred to as a double multivariate design. The design allowed for testing of general effects through multivariate analysis of variance (MANOVA) with repeated measures. However, the results from the repeated measure MANOVA analysis were vague, and to answer specific questions, it was necessary to simplify data into specific models that allowed for more detailed analyses.

There are 19 levels of the repeated measure dependent variable (19 total: 18 samples plus average of the four baseline samples) and four within subject factors (ACh, Asp, Glu, and GABA). There are four levels of the independent variable: saline/saline (SAL/SAL), AMN/saline (AMN/SAL), GB, and VX. Nested within two of those are two additional levels: GB S/NS and VX S/NS. Repeated measure ANOVAs were performed prior to each of the one-way ANOVA tests mentioned below. For each time point, one-way ANOVA was used to compare the treatment groups with respect to the NT concentrations.

To test for an AMN effect, SAL/SAL and AMN/SAL groups were compared directly via one-way ANOVA, separate from the other treatment groups. Nerve agent versus control group comparisons were then performed with the AMN/SAL group, excluding the SAL/SAL group, as there was no significant difference between the AMN/SAL and SAL/SAL groups.

To test for differences in the time-course concentration profile of each NT due to seizure caused by two different nerve agents, a one-way ANOVA was utilized with treatment (three levels: AMN/SAL, GB S, and VX S) as the independent variable and NT concentration as the dependent variables (19 total: 18 samples + average of the four baseline samples). Time-course concentration profiles for each NT were compared separately. Levene's test was used to test for homogeneity of variances, and it was necessary to perform a natural log transformation of the data to satisfy parametric assumptions. Multiple comparisons were run with Tukey's honestly significant difference (HSD) to identify the time points at which the treatment effect was significant.

The tests for differences in the time-course concentration profile of each NT, when there was no seizure caused by two different nerve agents, were identical to those described above for differences in seizure effects, but the three levels of the independent variable were AMN/SAL, GB NS, and VX NS.

To test for change in the time-course concentration profile of each NT due to the presence or absence of seizure activity after exposure to either nerve agent, a one-way ANOVA was utilized with treatment (three levels: AMN/SAL, nerve agent S, and nerve agent NS) as the independent variable and NT concentration as the dependent variables (18 samples plus average of the four baseline samples). Time-course concentration profiles for each NT were compared separately. GB and VX were compared separately. Levene's test was used to test for homogeneity of variances, and it was necessary to perform a natural log transformation of the data to satisfy parametric assumptions. Multiple comparisons were run with Tukey's HSD to identify the time points at which the treatment effect was significant.

Bivariate correlation analysis was used to test for a within subjects relationship between ACh and Glu. Data were split by treatment. The pivot table output allowed for examination of the relationship between levels of NTs at different time points, with the correlation between early ACh changes and later Glu changes of particular interest.

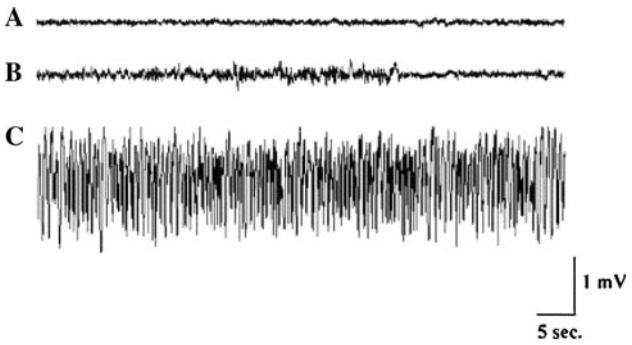

Fig. 2 Typical EEG recordings before and after nerve agent exposure. **a** Baseline EEG activity. **b** EEG in non-seizure group. There were small EEG disturbances; however, no seizure activity followed. **c** Status epilepticus in nerve agent-treated seizure animals

Results

Seizure occurrence and lethality

Not all animals developed continuous *status epilepticus* seizure following exposure to 1.0 × LD_{50} of GB or VX. Examples of baseline, non-seizure, and *status epilepticus* EEG recordings are displayed in Fig. 2a, b, and c, respectively. Rates of seizure occurrence were 66.7% (8/12) for GB and 37.5% (6/16) for VX; these rates were not significantly different. All VX animals survived for 24 h after exposure, whether they developed seizures or not, and all GB-exposed animals that did not develop seizures survived for 24 h also. However, for GB-exposed animals that developed seizures, lethality was 50% (4/8), a significantly higher rate than that of the VX seizure group ($\chi^2 = 4.2$, $df = 1$, $P < 0.05$). This seizure and lethality data are presented in Table 1. The mean times from exposure to seizure onset were 13 ± 0.9 and 48 ± 3.9 min (mean ± SEM) for GB and VX, respectively. These two onset times were significantly different.

Striatal ACh levels

Average basal ACh concentrations ranged from 0.05 to 1.92 pmol/10 µl of sample. Basal levels of the different treatment groups (SAL/SAL, AMN/SAL, GB, and VX) were not significantly different. No changes were observed in the SAL/SAL and AMN/SAL control groups during the entire collection period. Time-course changes in ACh relative to baseline for sustained seizure (S) and non-seizure (NS) groups exposed to GB and VX are displayed in Fig. 3a and b, respectively. The time of AMN administration is

Table 1 Summary of the numbers of animals with seizure activity and 24-h lethality after exposure to 1 × LD_{50} of GB or VX

GB		VX	
Seizure = 8 (67%)	No seizure = 4 (33%)	Seizure = 6 (38%)	No seizure = 10 (62%)
Lethality = 4 (50%)	Lethality = 0	Lethality = 0	Lethality = 0

referred to as −30 min, and the time of nerve agent exposure is referred to as 0 min. Data points are placed at the beginning of the collection time, so the average percent baseline value at 0 min (nerve agent exposure) represents samples collected between 0 and 15 min after nerve agent exposure.

According to repeated measure ANOVA, the GB S and GB NS groups were significantly different (Fig. 3a). The GB S group was significantly different from the AMN/SAL group between 30 and 90 min after exposure, and from the GB NS group between 30 and 120 min after exposure. Maximal increase in striatal ACh (417.1 ± 100.8%) occurred at the 45-min time point. The GB S group was also significantly different from the GB NS group during the first 15 min after exposure, which could indicate a small, localized AMN effect due to disruption of the blood–brain barrier or other factors during probing. However, considering the lack of significant difference between the AMN/SAL and SAL/SAL groups, and the diminutive size of the increase as compared to subsequent changes in ACh, it appears to be irrelevant. At no time was the GB NS group different from the AMN/SAL group.

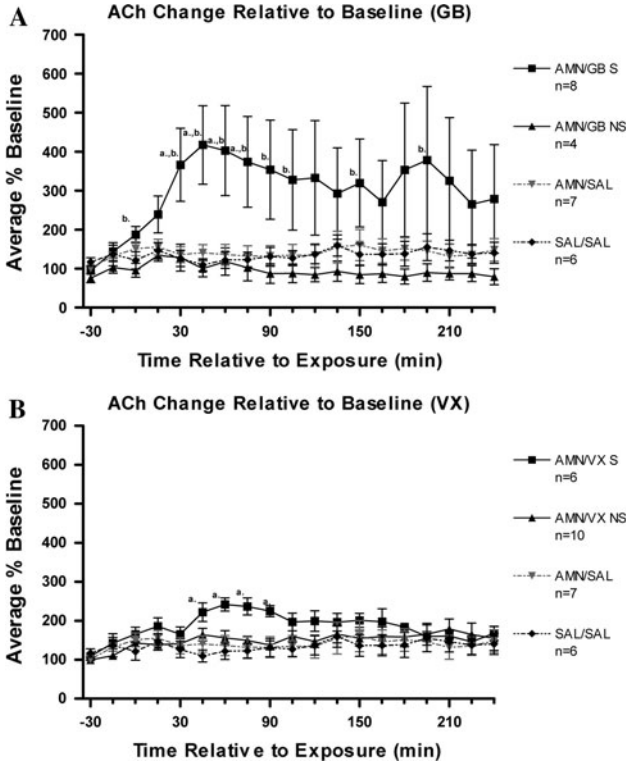

Fig. 3 Changes in extracellular striatal ACh concentrations after exposure to 1× LD_{50} of GB (**a**) or VX (**b**). Sustained significant ACh elevation distinguishes S from NS groups, and earlier ACh increases distinguish GB from VX groups. *Points* and *error bars* represent the mean of ACh concentrations taken as percentages of the baseline of each individual subject ± SEM. AMN was administered at −30 min, and nerve agent at 0 min. (*a.*, *b.*: $P < 0.05$ Agent S vs. AMN/SAL and Agent NS, respectively)

The VX S group was not different from the VX NS group overall (according to repeated measure analysis) or during the collection of any individual time point (Fig. 3b). The VX S group was different from the AMN/SAL group between 45 and 105 min after exposure. Maximal increase (241.9 ± 16.7%) occurred at the 60-min time point. At no time was the VX NS group different from the control group.

When GB S and VX S groups were compared directly, the GB S group was significantly greater at the 30-min time point. The GB NS and VX NS groups were not statistically different.

Striatal Glu levels

Average basal Glu concentrations ranged from 1.4 to 80.4 pmol/17 µl of sample/OPA mixture. Basal levels of the different treatment groups (SAL/SAL, AMN/SAL, GB, and VX) were not significantly different. No change from these basal levels was observed in the SAL/SAL and AMN/SAL control groups during the entire collection period. Time-course changes in Glu relative to baseline for S and NS groups exposed to GB are displayed in Fig. 4. One animal had an unrealistically high Glu concentration at a single sample time after exposure and this data point was eliminated from the analysis and graph. There were no significant changes in Glu levels in the VX S or VX NS treated animals. Again, data points are placed at the beginning of the collection time, so the average percent baseline value at 0 min represents samples collected between 0 and 15 min after nerve agent exposure.

According to repeated measure ANOVA, the GB S and GB NS groups were not significantly different. The GB S group was significantly different from the GB NS group between 180 and 195 min. Maximal increase

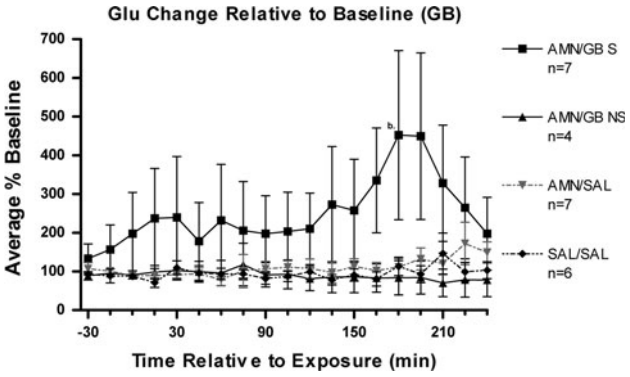

Fig. 4 Changes in extracellular striatal Glu concentrations after exposure to 1× LD_{50} of GB. Significant Glu elevation distinguishes GB S from NS groups. *Points* and *error bars* represent the mean of Glu concentrations taken as percentages of the baseline of each individual subject ± SEM. AMN was administered at −30 min, and nerve agent at 0 min. (*b.*: $P < 0.05$ Agent S vs. Agent NS)

(452.3 ± 218.5%) occurred at the 180 min sample time. At no time was the GB NS group different from the AMN/SAL control group.

The VX S group was not different from the VX NS group overall (according to repeated measure analysis) or during the collection of any individual time point. The VX S and VX NS groups were not different from the AMN/SAL control group at any time during the sample collection.

When GB S and VX S groups were compared directly, Glu concentrations in the GB S group were significantly greater than in the VX S group between 90 and 240 min post-exposure both with and without the outlier. The GB NS and VX NS groups were not different from each other.

There was a significant relationship between ACh at 45 min and Glu at 180 min post-exposure in the GB S group. Additional correlations were found to exist in the other treatment groups, but the above relationship was the only correlation involving points at which a significant increase in NT was observed.

Striatal Asp and GABA levels

Asp and GABA concentrations were often below detectable limits at basal levels with sporadic instances of increase. Asp and GABA were often elevated when Glu was elevated, but did not demonstrate any significant trends in response to nerve agent (data not shown).

Discussion

The purpose of this study was to analyze changes in the levels of the neurotransmitters ACh, Glu, Asp, and GABA following acute exposure to the nerve agents GB and VX. It was found that elevations in striatal ACh occurred only following the onset of seizure activity elicited by either nerve agent. In animals that did not develop seizures, ACh levels did not change in response to either GB or VX exposure. Levels of Glu showed a trend to increase following the onset of GB-induced seizures, but did not change following VX-induced seizures, while concentrations of Asp or GABA were unaffected by either nerve agent under these conditions.

Seizure activity began significantly earlier (13 min) after GB exposure when compared to the protracted onset of seizures (48 min) following VX exposure. These differences in time for development of central signs of nerve agent toxicity are consistent with a previous ACh study (O'Donnell et al. 2010a), as well as time-course comparisons of GB and VX AChE inhibition in the striatum (Shih et al. 2005). Animals exposed to GB experienced rapid and severe cholinergic toxicity compared with the VX group, which is in agreement with the observed seizure onset times with these two nerve agents (this study and Shih et al. 2003, 2007). While GB-exposed animals experienced lethality, this occurred only in the animals that developed seizures. This close association between nerve agent-induced lethality and seizure activity has been observed in previous studies (O'Donnell et al. 2010a, b).

The highly variable and slow increases in Glu concentrations that developed in response to GB-induced seizures and the failure of VX-induced seizures to elicit any changes in striatal Glu concentrations were somewhat surprising. Increases in extracellular Glu concentrations have been consistently reported to occur in diverse limbic brain areas (CA_1, CA_3, dentate gyrus, amygdala, piriform cortex, septum) following the onset of seizures elicited by the nerve agent soman (Lallement et al. 1991a, b, 1992b; Wade et al. 1987). Both the magnitudes and time-courses of these reported Glu increases differed depending upon the brain region studied. Failure to see a similar robust increase in Glu in this experiment may be due to the relatively low density of glutamate synapses in the striatum, which in turn might explain the fact that there is a low degree of neuropathology observed in striatum following nerve agent-induced seizures (McDonough et al. 1998, 2000).

Extracellular Asp or GABA concentrations did not follow a consistent change in time-course profiles among subjects within the respective treatment groups. In contrast, Wade et al. (1987) reported large increases in extracellular Asp concentrations in the piriform cortex following GD-induced seizures, while Lallement et al. (1991a) reported no change in Asp in several areas of the hippocampus during similar seizures. This same group also reported no change in extracellular GABA levels in hippocampus during the first 30 min following GD-induced seizures in rats (Lallement et al. 1993). Thus, changes in Asp and GABA may be regionally specific and/or do not respond to seizures elicited by these nerve agents. In conclusion, toxic characteristics of GB and VX, as well as the relationships between seizure occurrence, ACh increase, Glu increase, and lethality have been examined. Seizure onset times and timing of cholinergic toxicity were consistent with delayed passage of VX across the blood–brain barrier due to its larger size and protonated structure in vivo. Observations indicated that, at a given dose, a nerve agent's propensity for initiating seizure is not proportional to lethality among subjects experiencing seizure. As in previous studies with other nerve agents (O'Donnell et al. 2010a, b), ACh levels in the striatum increased following the triggering of seizure activity by these nerve agents, again indicating that this ACh response is the result of the seizure activity. Changes in Glu levels were confined to animals exposed to GB that developed seizures, while there were no reliable changes in levels of Asp or GABA.

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Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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